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Mukundan (foreground) with four members of her team: (left to right) Zachary Stromberg, Loreen Stromberg, Kiersten Lenz, and Laura Lilley.

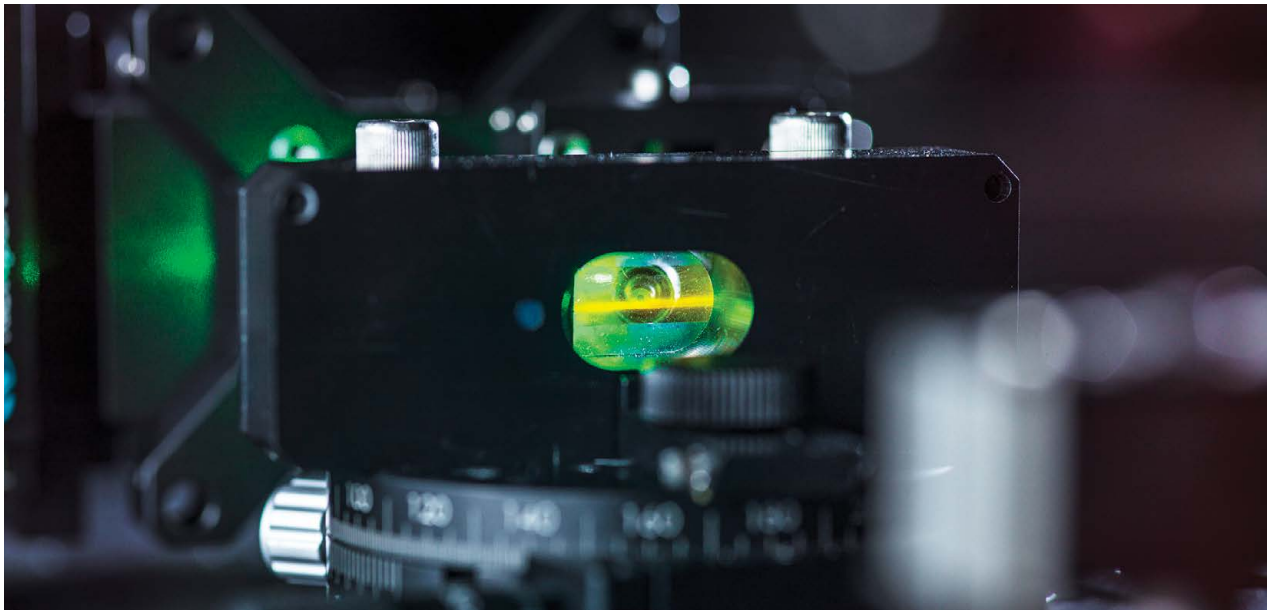
Reinventing Infection Detection

Microbiologist **Harshini Mukundan** relies on the chemistry of the immune system to diagnose disease quickly and accurately.

WHEN I VISITED KENYA IN 2016 as part of a new research collaboration, I took chocolate, candies, and toys to share with the children at the medical clinic and the local village. Growing up in rural areas, these children have limited exposure to the delicacies of the Western world—things that we sometimes take for granted—and bringing treats was a small, easy gesture that could put a smile on their faces. But my visit was part of a larger gesture—something my collaborators and I have been working on for many years—an effort to develop technologies to keep children healthy with increased access to medical advances.

Access to medical care is limited in resource-poor areas of the world, and families often travel great distances to seek treatment. Because of the economic burden, most families are unable to make multiple visits to the doctor. Hence, it is critical that the medical providers be able to diagnose and treat an infection right then and there at the point of need. This requires simple and effective diagnostic platforms that can give useful answers right away—answers that enable providers to respond quickly and to reliably dispense their limited supply of medications to the most needy patients.

First and foremost, medical professionals need to know: Is the causative agent bacterial or viral? And if possible, what specific pathogen is it? The challenge in addressing these questions is that there is no single test that can be used to diagnose all infections, as most diagnostic tools target only one specific type of pathogen. This technology gap forces clinicians to choose a diagnostic test based on details of the patient's symptoms—fever, congestion, vomiting—combined



The optical waveguide sensor is a rapid, sensitive method for detecting certain molecules within a complex sample. Mukundan and her team have been adapting and miniaturizing the sensor platform for use in remote areas to detect bacterial pathogens.

with what diseases are currently prevalent in the area and what tests are available and affordable. Unfortunately, this so-called prior knowledge of the possible causes of disease inadvertently causes a bias in the diagnosis. Overcoming this situation and developing a universal diagnostic tool is my dream—and the challenge towards which our team has been working for the past decade. I am excited to say that we are making progress! We now have a prototype that can quickly screen samples for any type of pathogen—eliminating the need for prior knowledge—and that would be useful for doctors in first and third world countries as well as for veterinarians and even military personnel in rural areas who may not have access to extensive laboratory capabilities.

I get to tell this story, but the credit behind this progress goes to an incredible team of individuals at Los Alamos and collaborating institutions—a team of microbiologists, molecular biologists, immunologists, engineers, theorists, statisticians, chemists, and physicists who have worked with me toward this dream.

Immunity is innate

Growing up in India, I suffered from mumps and watched my sister battle measles. I was fascinated with infectious diseases and how the interaction between our immune systems and pathogens determines whether we suffer from a disease, defeat it, or succumb to it. As a high school student, my mandatory voluntary work—labeled "social useful productive work"—introduced me to tuberculosis patients at the local health center, where I learned that their treatment regimens had to be monitored for a grueling six months to ensure success. These events led me to think a lot about disease and medicine, which motivated me to study microbiology at university and pursue graduate studies in biomedical sciences in the United States.

I came to Los Alamos in 2006 as a postdoctoral researcher after finishing my Ph.D. in Biomedical Sciences at the University of New Mexico. My mentor, Basil Swanson, had been working on the development of a detection technique called a waveguide-based optical biosensor for the rapid detection of environmental organisms. Optical waveguides are small translucent plates made of two materials that differ in their ability to refract light. Propagation of light through the waveguide generates an optical field called an evanescent field, the intensity of which falls sharply as the distance from the waveguide increases. Thus, unlike in other platforms, the optical field is effectively confined to the surface of the waveguide where target molecules can be bound; this is an advantage because it eliminates the possibility of detecting extra "contaminant" molecules that may be present in a complex sample (other platforms would require additional steps to wash away these extra molecules). The optical waveguide confers excellent sensitivity and speed, although it does not add to the specificity of detection. However, when used in conjunction with fluorescently tagged molecules of interest (because the field is strong enough to excite the tags when they are bound to the waveguide surface), the combined detection technique is quick and effective.

I was awarded a National Institutes of Health postdoctoral research fellowship to explore the adaptation of this sensor technology toward the development of diagnostics for tuberculosis, one of the oldest and most challenging diseases known to man. Therein began my journey of trying to develop diagnostics. I was fortunate to have excellent mentors, collaborators, team members, and advisors—all of whom facilitated learning and advancement down this path.

During this time period, my infant nephew got sick with meningitis. Viral meningitis is self-limiting and patients usually

recover from it in about 10 days without treatment, whereas the bacterial form of the disease often requires extensive antibiotic treatment. To my surprise, I learned that there were no diagnostic tests to differentiate between the viral and bacterial disease. Thankfully, my nephew recovered and is now a strapping teenager. He did not require many rounds of antibiotics, so it is probable that he had the viral form and the antibiotics were not necessary. Yet the fact that he was treated with antibiotics simply because they could not differentiate the correct causative agent nagged at me, since unnecessary use of antibiotics can lead to antibiotic resistance. This led me toward a desire to develop a more universal diagnostic platform to discriminate bacterial infections from viral ones.

In our quest to develop such a platform, my team and I looked to the human immune system for inspiration. The human immune system has two parts: adaptive and innate. The adaptive system is well known for its development of antibodies that "remember" pathogens they've encountered before. On the other hand, the innate system is able to recognize and mount an immune response against invading pathogens effectively and quickly without any prior exposure to the specific pathogen. The innate immune system accomplishes this using a network of molecules that distinguish cells that belong to our bodies ("self") from foreign cells that don't belong ("non-self"). Some non-self molecules exist because they were released by the pathogen into the host during the course of infection. These molecules, known as biomarkers, are extremely consistent (or "conserved") across multiple pathogen species, and as a result, human immune receptors recognize them all as disease. Simply put, whether it is antibiotic-resistant tuberculosis (TB) or a newly emerging strain of staphylococcus, the innate immune system only needs to detect that one of these types of biomarkers is present to mount a response.

With this in mind, my team and I wanted to understand more about these conserved biomarkers and unravel the mechanisms by which they interact and associate with the human host. If we could mimic innate immune recognition in the laboratory, we could—in theory—repurpose it for a universal diagnostic strategy. Such a method would not require the user to have prior knowledge of what the pathogen could be, would not be driven by the patient's symptoms, could be applied for existing and emerging infections with equal efficacy, and could provide early diagnostic information to guide decision making and suitable therapeutic intervention.

We started by working on such a strategy for the diagnosis of bacterial infections, specifically TB.

LAM and the biological taxi service

Tuberculosis, caused by the bacterium *Mycobacterium tuberculosis*, is one of the oldest diseases known to infect humans, yet it is notoriously difficult to diagnose. With the evolution of drug resistance, and the recent phenomenon that tuberculosis is often associated with HIV infection, the diagnostics problem has become even more severe in the past few decades.

Several investigators have considered detecting one of these aforementioned biomarkers: a molecule called lipoarabinomannan (LAM) that appears in patients with an active TB infection. A component of the bacteria's cell membranes, LAM is a lipidated sugar, or lipoglycan, meaning that it has a sugary part and a fatty part. When *M. tuberculosis* cells are engulfed by some of our immune cells (macrophages) in the lung, the TB cells encounter innate immune receptors. LAM is known as a virulence factor because it activates the immune receptors in this environment, and therefore direct measurement of LAM in infected patients can provide an effective strategy for diagnosing active TB.

Several investigators (including us) have developed tests to measure LAM in urine, but uric acid can break apart the LAM molecule, so detecting it in blood would be better—albeit more difficult. Upon investigation, my team and I realized that the difficulty was directly owing to the biochemistry of LAM. LAM is an amphiphile, meaning the lipid part is hydrophobic (water-repelling) and the sugar part is hydrophilic (water-loving). Therefore, like oil droplets that group together in water, hydrophobic molecules find each other rather than float around freely in aqueous blood. However, because the human body is composed of many hydrophobic lipids that often need to travel in blood, our bodies have lipid-carrying molecules, called lipoproteins, whose job it is to transport lipids from one part of the body to another, behaving as a "biological taxi service." Examples of these courier molecules include high-density and low-density lipoproteins (HDL and LDL), which are commonly known as indicators of cardiac health. My team and I wondered if these lipid couriers also transported pathogen biomarkers in blood, and exploration of this hypothesis resulted in a resounding "yes"! HDL and LDL function as carriers for both host and pathogen lipids through the blood.

In our quest to develop a universal diagnostic platform, we looked to the human immune system for inspiration.

Upon further study we learned that although LAM is a biomarker for TB, most bacterial pathogens secrete other kinds of lipidated sugar biomarkers that are involved in virulence and immune recognition. For instance, one class of bacteria, called gram negative, releases lipopolysaccharides (LPS), and another class, gram positive, releases lipoteichoic acid (LTA). The host's HDL, LDL, and other lipoprotein molecules transport these biomarkers around.

Unraveling this concept of host-pathogen interactions helped solidify a strategy for my team: Using LAM, LPS, LTA, and other biomarkers, we developed two detection assays by capitalizing on their association with HDL and LDL carrier molecules. This is a relatively novel approach because many current diagnostics instead rely on detecting protein-based molecules that are hydrophilic and thus easily found in blood or urine.

Our first assay, lipoprotein capture, tested the concept of using lipoproteins but in a way that requires prior knowledge of the target pathogen, making this assay most useful for clinical research and animal studies. Based on the success of capturing and detecting lipoproteins, we developed our second assay, membrane insertion, to be a truly universal diagnostic approach:

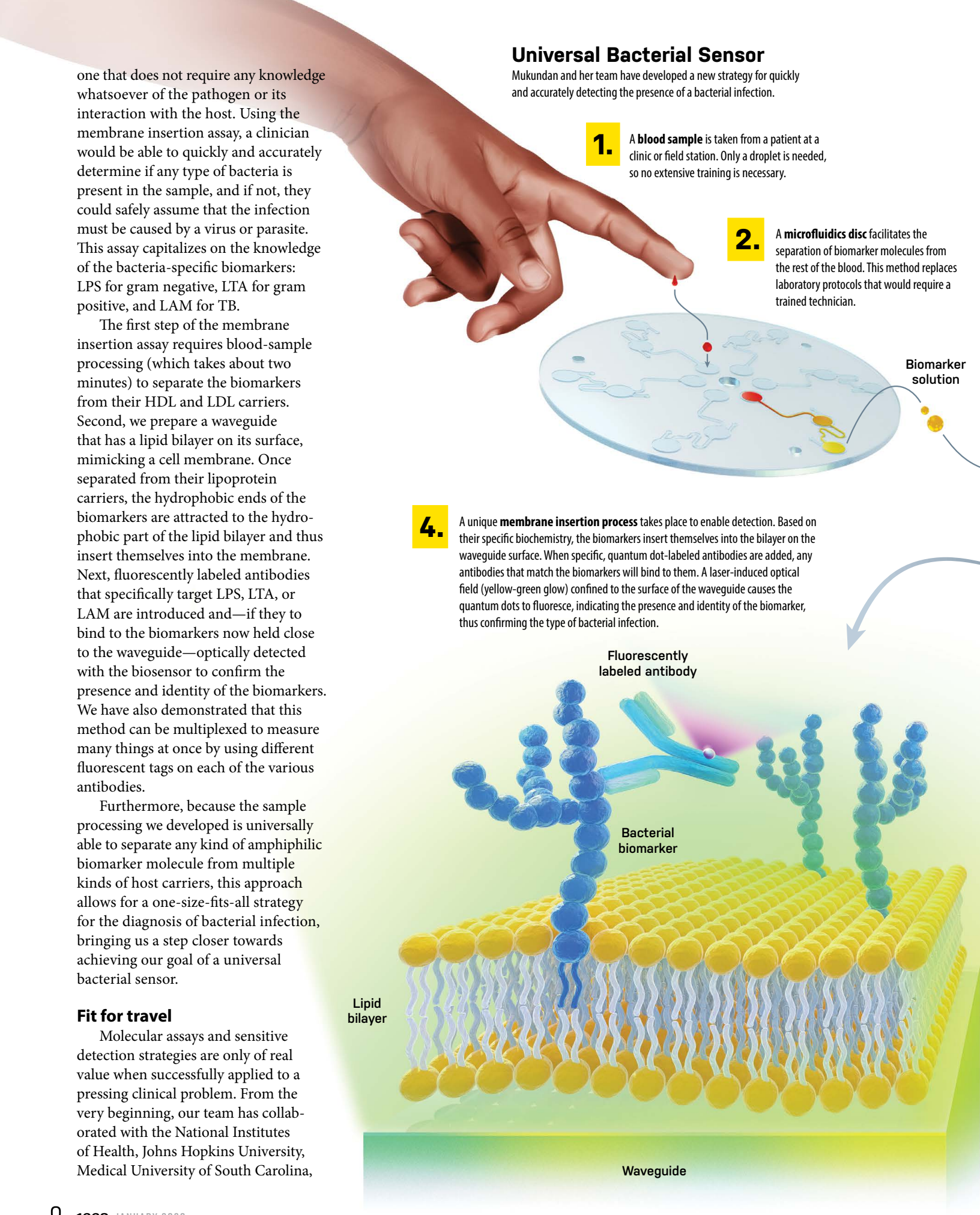
one that does not require any knowledge whatsoever of the pathogen or its interaction with the host. Using the membrane insertion assay, a clinician would be able to quickly and accurately determine if any type of bacteria is present in the sample, and if not, they could safely assume that the infection must be caused by a virus or parasite. This assay capitalizes on the knowledge of the bacteria-specific biomarkers: LPS for gram negative, LTA for gram positive, and LAM for TB.

The first step of the membrane insertion assay requires blood-sample processing (which takes about two minutes) to separate the biomarkers from their HDL and LDL carriers. Second, we prepare a waveguide that has a lipid bilayer on its surface, mimicking a cell membrane. Once separated from their lipoprotein carriers, the hydrophobic ends of the biomarkers are attracted to the hydrophobic part of the lipid bilayer and thus insert themselves into the membrane. Next, fluorescently labeled antibodies that specifically target LPS, LTA, or LAM are introduced and—if they to bind to the biomarkers now held close to the waveguide—optically detected with the biosensor to confirm the presence and identity of the biomarkers. We have also demonstrated that this method can be multiplexed to measure many things at once by using different fluorescent tags on each of the various antibodies.

Furthermore, because the sample processing we developed is universally able to separate any kind of amphiphilic biomarker molecule from multiple kinds of host carriers, this approach allows for a one-size-fits-all strategy for the diagnosis of bacterial infection, bringing us a step closer towards achieving our goal of a universal bacterial sensor.

Fit for travel

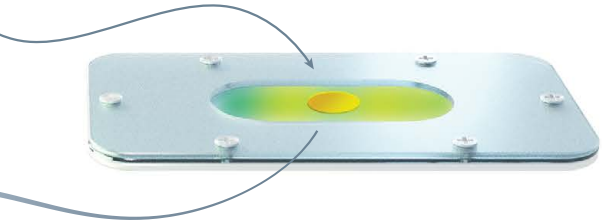
Molecular assays and sensitive detection strategies are only of real value when successfully applied to a pressing clinical problem. From the very beginning, our team has collaborated with the National Institutes of Health, Johns Hopkins University, Medical University of South Carolina,



and other institutions to adapt our assays to the clinical scene. In 2015, theoretical biophysicist Ben McMahon and I, along with others in the Los Alamos Theoretical and Bioscience divisions, began a collaboration with Dr. D. J. Perkins and his team at the University of New Mexico Center for Global Health to evaluate our diagnostic methods with pediatric clinical samples from a resource-poor clinic in Siaya, Kenya. This collaboration has proved invaluable and fruitful in many ways.

In sub-Saharan Africa, a leading cause of childhood mortality is bacteremia, also known as sepsis: the condition when bacteria are present in the bloodstream. Unfortunately, this condition (bacteremia itself is not a disease) is often difficult to diagnose; although microbial culture is the gold standard, it is slow and not particularly sensitive. In addition, patients often have co-infections such as malaria or HIV that can complicate the diagnosis. Our universal sensor could help these clinics by enabling them to easily screen patients for bacterial infection—providing diagnostic information in a timely manner to guide treatment. However, a few things needed to be modified for our methodology to be applicable in this scenario.

3. The biomarkers are added to a **waveguide** that has been prepared with a lipid bilayer to mimic the membrane of a cell.



We want to make it easy for clinicians to determine if any type of bacteria is present in the sample.

For one, although our processing steps to separate the serum and biomarkers from the blood are quick, they require laboratory infrastructure not readily available in resource-poor areas such as rural Africa. To resolve this, we have been working on developing a microfluidics approach, which will enable rapid, lab-free sample processing at the point of need. Collaborating with experts in the Physics and Bioscience divisions at Los Alamos, we have designed and validated a microfluidics disk and are currently integrating it with the sensor platform. This is the first time a microfluidics approach has been used for lipid extraction from blood, and the outcome stands to be a simple and safe system that could facilitate the deployment of our sensing platform.

In addition to optimizing the sample preparation strategy, we also needed to re-evaluate the waveguide-based sensor platform. The waveguide platform has provided greater detection sensitivity compared to conventional methods in all our previous evaluations; however, it was originally developed over a decade ago and is not suitable for use in the field. Therefore, we decided to simplify and reengineer the platform to make it travel-friendly. In collaboration with scientists from the Physics Division and engineers at the Los Alamos National Security Education Center, we have now successfully miniaturized the instrument and developed a portable version; we are currently working on evaluating it and optimizing its sensitivity and performance.

The whole idea is to have one box that anyone can use at the point of need: a primary care doctor or specialist, a soldier, an emergency responder, or a veterinarian. And I'm happy to say we're getting close to having one.

Lessons in life and science

Working on this biosensor has taught me a number of things about chemistry and medicine and has exposed me to new challenges in optics, engineering, and informatics. But beyond the science, this effort has taught me a great deal about teamwork. Bringing the universal bacterial sensor to this point of development has required input from engineers, physicists, chemists, informatics experts and theorists, biologists and microbiologists, clinicians, molecular biologists, and veterinarians—a truly multi-disciplinary collaborative effort. The project has allowed each of us the opportunity to learn something new, to contribute something to the final product, and to be part of a wonderful team. The team has also included students, postdoctoral fellows, technologists, and engineers—each of whom has a unique perspective that helped shape the science. Not only did the team demonstrate intellectual diversity, but also social diversity—involving individuals from multiple social, ethnic, and gender backgrounds from various countries, including a team in Kenya that has played a critical role in the clinical recruitment and evaluation.

This article may be focused on my impressions—but these impressions and thoughts are made possible by the enthusiastic and passionate participation of members of this team. As a team, we hope to make an impact on diagnostics in the future. Our goal—indeed, our dream—is to remove any element of guesswork from the diagnosis of infectious disease and to have specific and rapid identification of a pathogen at the point of need. Realizing this dream is certainly a work in progress that has had many disappointments and derailments along the way. But there are two lessons I like to live by: One is to never give up on the things you really want in life, and the other is to not be afraid to change direction when something does not go the way you planned or envisioned.

So onward we go, without giving up, changing direction as needed along the way. One of the best aspects of this journey is that it is so easy to be inspired by the children in Kenya. These little ones are by far the most positive and cheerful individuals I have encountered in my life. On one of these visits, I would like to take something more than chocolates: the hope for a healthy future. LDRD

—Harshini Mukundan